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TITLE: Construction of a Vesicular Stomatitis virus Expressing Both a Fusogenic Glycoprotein and IL-12: A Novel Vector for Prostate Cancer Therapy

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Introduction: Vesicular Stomatitis Virus (VSV) infection of malignant cells results in oncolysis, sparing normal cells due to inherent differences in the interferon response pathway. In this study we explored enhancing VSV-G by engineering it to express the fusogenic glycoprotein from the Newcastle Disease Virus (VSV-F) to induce inter-cellular membrane fusion producing syncytia, which are incompatible with cell survival. Materials and Methods: Studies initially compared effects of VSV and VSV-F in vitro in prostate cancer cells lines, noting differential effects at different cell densities and the induction of apoptosis. Studies then compared effects of VSV vs VSV-F in a orthoptic mouse model of prostate cancer, focusing on tumor size at set time points and survival. Results: As the confluence of cells in the wells became greater, VSV-F showed more rapid cell kill than VSV-G (p<0.001). VSV-G mediated growth suppression by inducing apoptosis; this effect was slightly attenuated in VSV-F. In both single and serial viral injections VSV-F resulted in significant survival enhancement over control and VSV-G. Interestingly. Repetitive injections of VSV-G was no better than a single injection. Mechanistic studies suggested that some prostate cancer cell lines do not have as attenuated IFN response pathways, which can be over come by the high levels of IFN found within injected tumors. Discussion: Through the induction of the fusogenic protein, VSV-F maintains superior growth control of only moderately IFN responsive cell lines, most likely through an induced immune response.

15. SUBJECT TERMS
Cancer Therapy: VSV, fusogenic glycoprotein, syncytia, oncolysis

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Introduction

The goal of this grant proposal is to examine the efficacy of an engineered VSV that expresses the fusogenic protein from the Newcastle Disease Virus (NDV) in comparison to wild type VSV. Fusogenic protein expression has been shown to lead to necrotic cell death through the production of large syncytia, which in turn may result in anti-tumor immune effects. Our efforts focused on a mutated fusogenic glycoprotein, L289a, which allowed for syncytia formation independent of the viral hemagglutinin-neuraminidase (HN) protein and exhibits a 50% enhancement in HN-dependent fusion over wild-type (wt) F protein. Through the introduction of syncytia formation, we proposed that we would engender better cell death in prostate cancer cell lines when compared to native VSV, while retaining its specificity for cancer cells and spare normal cells. Work during the first year demonstrated that both VSV and VSV-F in the presence of low doses of interferon did not kill normal prostate epithelial cells, while under the same conditions rapidly killed most prostate cancer cells in vitro. In each instance cell kill was seen even at low initial doses of virus and VSV-F demonstrated superior efficacy over wild-type VSV (VSV-G). This past year studies focused on completion of some in vitro studies and

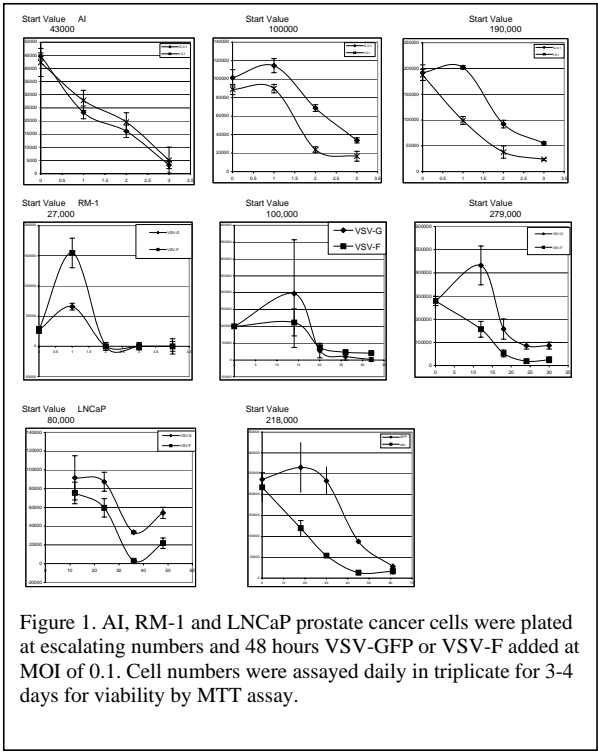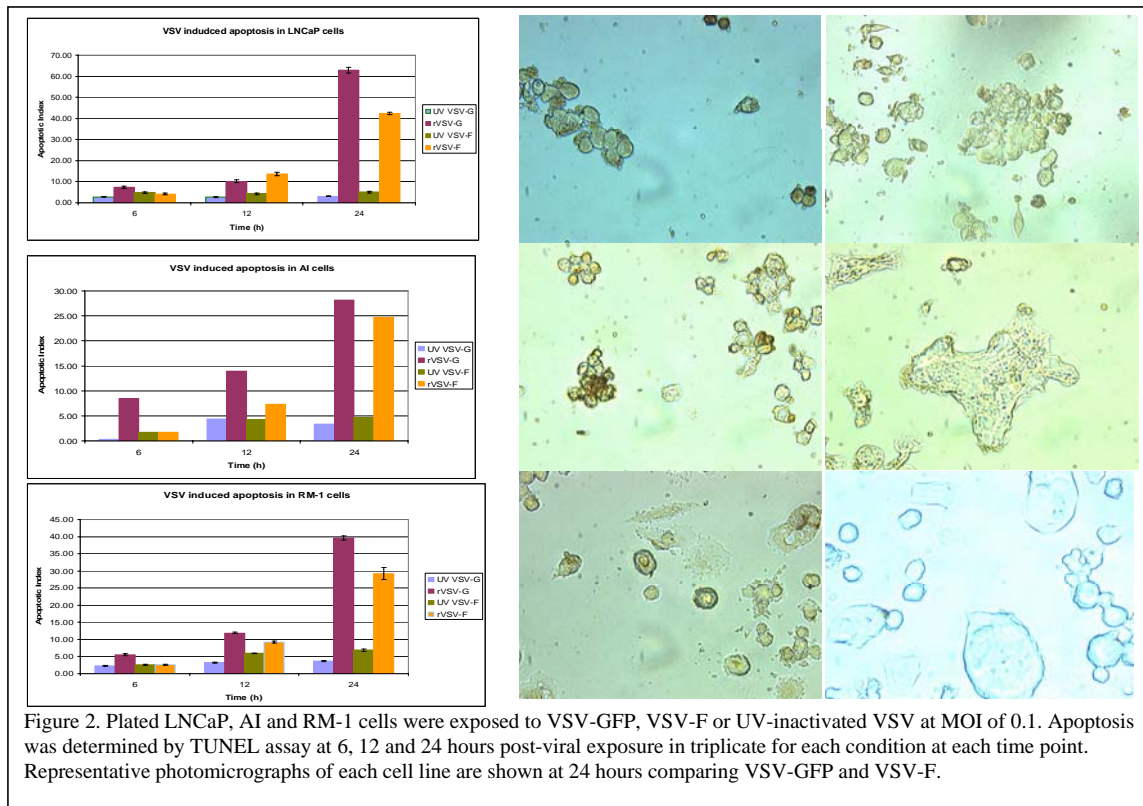exploration of the in vivo effects of VSV versus VSV-F.

Figure 1. AI, RM-1 and LNCaP prostate cancer cells were plated at escalating numbers and 48 hours VSV-GFP or VSV-F added at MOI of 0.1. Cell numbers were assayed daily in triplicate for 3-4 days for viability by MTT assay.

Body

Earlier studies demonstrated enhanced ability of VSV-F to a variety of prostate cancer cell lines. We then explored some of the underlying mechanisms. First, we explored the effect of varying plating densities of cells prior to virus exposure on the killing abilities of both viruses. As shown in Figure 1, the superior abilities of

Figure 2. Plated LNCaP, AI and RM-1 cells were exposed to VSV-GFP, VSV-F or UV-inactivated VSV at MOI of 0.1. Apoptosis was determined by TUNEL assay at 6, 12 and 24 hours post-viral exposure in triplicate for each condition at each time point. Representative photomicrographs of each cell line are shown at 24 hours comparing VSV-GFP and VSV-F.

VSV-F were more apparent at the higher densities. This finding would support the notion that the enhanced effects of VSV-F are mediated through formation of syncytia which are more likely to form at higher cell densities. Published experience with VSV has demonstrated its ability to kill through induction of apoptosis. We studied whether or not this characteristic remained in the face of expression of the F protein and the induction of syncytia, which mediate necrotic cell death. As demonstrated in Figure 2a, there was marked induction of apoptosis mediated by both viruses in all 3 cell lines peaking at 24 hours post-virus exposure. In each instance VSV-G induced more apoptotic activity, though this was a modest difference. By light microscopy at 24 hours, VSV-F induced syncytia, a phenomenon not seen with VSV-G. TUNEL staining of these syncytia was negative (data not shown). Therefore, it would seem that VSV-F retains the powerful ability to induce apoptosis in addition to killing cells through syncytia formation.
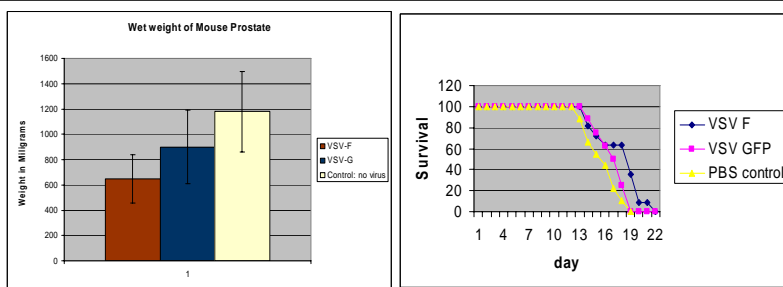
Figure 3. RM-1 orthotopic tumors were injected with VSV-GFP, VSV-F or PBS. Mice were either sacrificed at 14 days post-tumor cell inoculation or continued in a survival study. Tumors from sacrificed mice were weighed.

Our attention then turned to the performance of in vivo studies, using the orthotopic RM-1 model. Tumor cells are inoculated into the dorsolateral prostate and 6 days later macroscopic tumors were injected with virus. The maximum liquid volume that can be injected into the mouse prostate without gross spillage is 50ul; this correlated with $8x10^8$ vp which served as the dose tested. Mice were randomized to injection with a single therapeutic dose of VSV-G, VSV-F or equal volume of PBS. In the initial experiments all mice were sacrificed 8 days later, 14 days post-tumor inoculation, and wet weights of tumor obtained. Both vectors resulted in smaller tumors, 28% for VSV-G and 44% for VSV-F than control, respectively (Figure 3A) (p=0.03 VSV-G vs VSV-F t-test). As survival study was set up under the same conditions. Animals treated with VSV-F lived 25% longer than controls (Figure 3B) (19.5+/-1 days vs 15+/-.5 days, p<0.001, Mantel-Cox), while animals treated with VSV-G had median survival of 18+/-.45 days (VSV-G and VSV-F, p=0.001, Mantel-Cox). We then explored the potential benefit of repetitive injections of
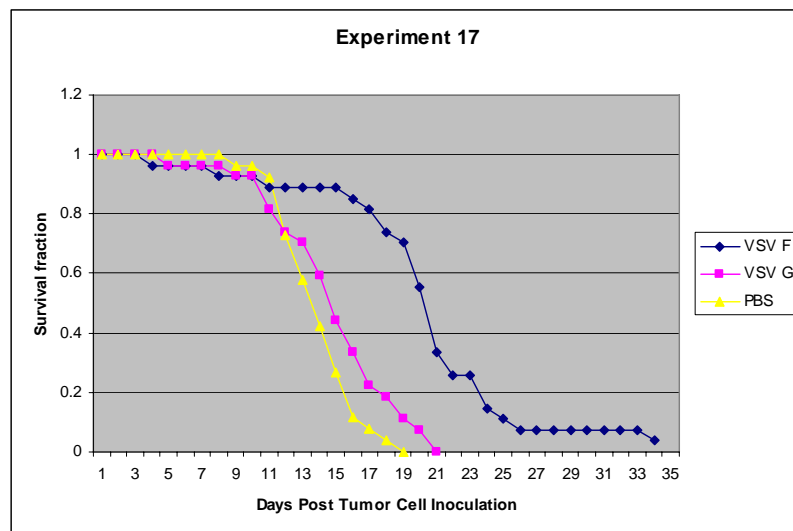

Figure 4. RM-1 Orthotopic tumors were injected with times with either VSV-GFP, VSV-F or PBS in a survival study.

VSV vectors. In a survival study mice were injected at days 6, 9 and 12 with $8x10^8$ vp. Median survival for VSV-G was 16.2+/-.5 days compared to 14.3+/-.5 days (Figure 4) (p=0.001,
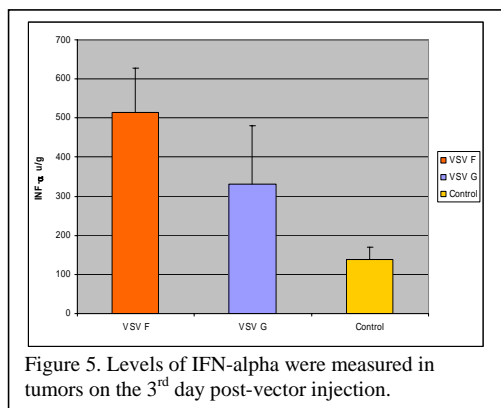
Mantel-Cox), compared to 23.6+/-2.3 days with 2 of 16 mice long term survivors.

Figure 5. Levels of IFN-alpha were measured in tumors on the 3rd day post-vector injection.

Interestingly, we noted that there was no enhanced benefit to 3 injections of VSV over a single injection. Among the possibilities reviewed was that perhaps the defective interferon response pathways could be overcome at higher doses of INF induced by the immune r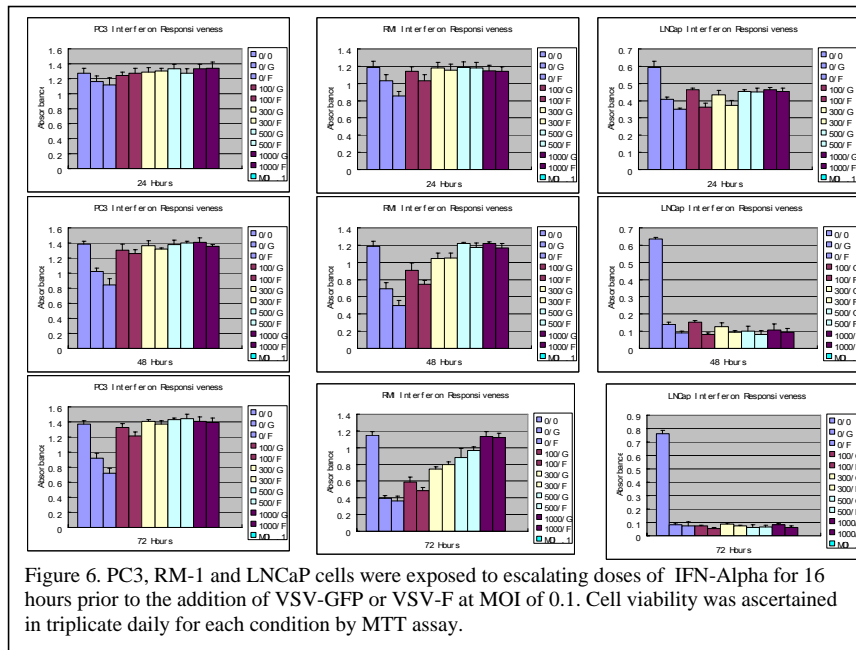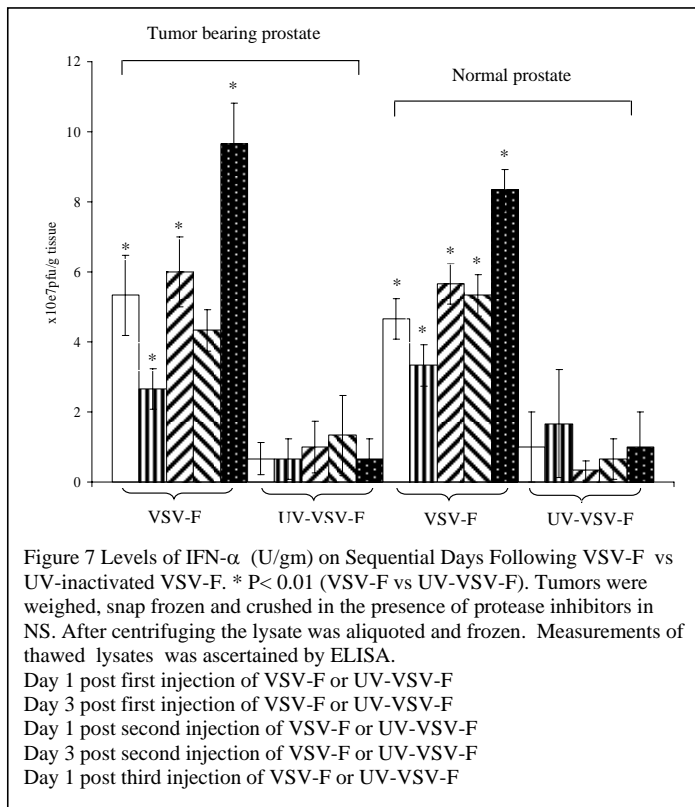esponse against the vector to negatively impact viral replication. To address this hypothesis, we first ascertained levels of IFN-α in treated tumors on the third day following vector injection – the day on which in the repetitive injection study, the second dose of virus would be injected.

Figure 6. PC3, RM-1 and LNCaP cells were exposed to escalating doses of IFN-Alpha for 16 hours prior to the addition of VSV-GFP or VSV-F at MOI of 0.1. Cell viability was ascertained in triplicate daily for each condition by MTT assay.

Tumors were excised and weighed, flash frozen and mechanically lysed in the presence of protease inhibitors. The level of IFN-α was measured by ELISA with the calculation taking into account the weight of the tumor. Levels of cytokine in VSV-G treated tumors were double that of control while levels in VSV-F tumors were three times higher than control (Figure 5). To explore how IFN-α would impact on VSV replication, we exposed 3 cell lines, PC3, RM-1 and LNCaP at a fixed dose of VSV-G and VSV-F (0.1 MOI) and escalating doses of cytokine: 0-

Figure 7 Levels of IFN-α (U/gm) on Sequential Days Following VSV-F vs UV-inactivated VSV-F. * P< 0.01 (VSV-F vs UV-VSV-F). Tumors were weighed, snap frozen and crushed in the presence of protease inhibitors in NS. After centrifuging the lysate was aliquoted and frozen. Measurements of thawed lysates was ascertained by ELISA.
Day 1 post first injection of VSV-F or UV-VSV-F
Day 3 post first injection of VSV-F or UV-VSV-F
Day 1 post second injection of VSV-F or UV-VSV-F
Day 3 post second injection of VSV-F or UV-VSV-F
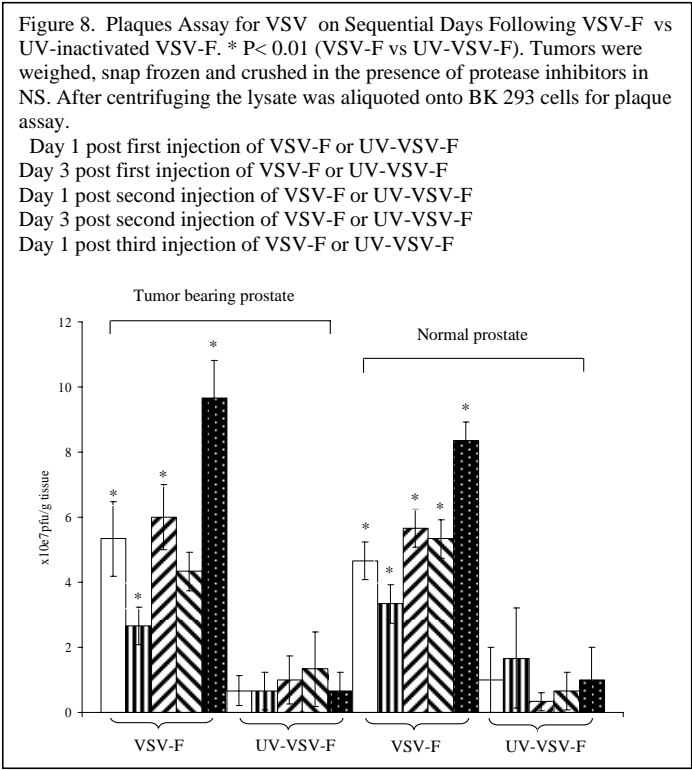Day 1 post third injection of VSV-F or UV-VSV-F

1000u (Figure 6). These studies noted differing responses to VSV infection: PC3 cells are relatively resistant to either VSV virus, which can be blocked by low doses of IFN- α, reminiscent of how non-malignant cells react. In contrast LNCaP cells remain sensitive to VSV infection and replication even at very high doses of IFN- α, demonstrating the presence of fully defective IFN response pathways. RM-1 cells are sensitive to VSV at low doses of IFN- α, which can be overcome by higher doses of cytokine, implying only partially defective pathways. Indeed, at the levels of IFN-α measured within the tumor VSV-G is unable to replicate and kill RM-1 cells in vitro. We felt that the lack of improved efficacy of repetitive injection of RM-1 tumors in vivo was due to the induction of high levels of IFN-a within treated tumors which inhibited replication of VSV.

To explore this hypothesis in vivo, tumor bearing were serially injected as set up in Figure 4 in addition to normal mouse prostate as a control and serially sacrificed at set time points following vector injection. Tumors were divided for measurement of cytokine levels or for performance of plaque assay to ascertain the level of virus within tumor tissue (Figure 7 & 8). We noted escalating concentrations of IFN with each injection in both tumor bearing and normal tissues not seen in control injected tissues due to the inflammatory response evoked in response to the vector injection (Figure 7). By plaque assay repetitive

dosing resulted only in increasing viral doses in normal prostate tissues, while there significantly less virus in prostate cancer tissue following the 3rd injection. Taken together this experiment validates our hypothesis that repetitive injections into tumor tissue results in elevated levels of IFN which in turn inhibits VSV replication and negates the therapeutic benefit of repeated injections. Furthermore, this clearly indicates the benefit seen with repeated injections with VSV-F must be related to the expression of the syncytial protein, presumably via an immune response as opposed to viral replication. Lastly, these studies indicate that theory that malignan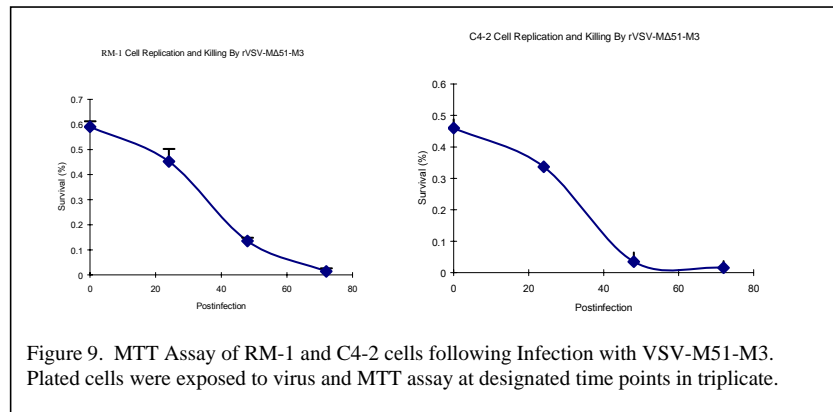t cells harbor clear abnormalities in the interferon response pathway is not universal and the future use of these virus will depend on further improvements in its engineering.

Figure 8. Plaques Assay for VSV on Sequential Days Following VSV-F vs UV-inactivated VSV-F. * P< 0.01 (VSV-F vs UV-VSV-F). Tumors were weighed, snap frozen and crushed in the presence of protease inhibitors in NS. After centrifuging the lysate was aliquoted onto BK 293 cells for plaque assay.
  Day 1 post first injection of VSV-F or UV-VSV-F
Day 3 post first injection of VSV-F or UV-VSV-F
Day 1 post second injection of VSV-F or UV-VSV-F
Day 3 post second injection of VSV-F or UV-VSV-F
Day 1 post third injection of VSV-F or UV-VSV-F

The Original application and SOW were to explore the ability of co-expression of the Fusion protein with IL-12 to enhance the expected immune induced by expression of the F protein independently despite numerous attempts. However, we were unable to construct a VSV vector which could express both IL-12 and NDV-F. Furthermore, given the problems of innate immunity reducing the ability of VSV to replicate in moderately sensitive cancer cells, the decision was made in a deviation from the SOW for the last year to construct a VSV vector which could evade the innate immune system.

Studies by others have demonstrated that cellular components of the innate immune system, such as granulocytes, natural killer (NK) cells, NKT cells, and macrophages, are rapidly recruited and activated at the

Figure 9. MTT Assay of RM-1 and C4-2 cells following Infection with VSV-M51-M3. Plated cells were exposed to virus and MTT assay at designated time points in triplicate.

sites of viral infection. These cells participate in the antiviral response both by directly killing infected cells and by producing antiviral cytokines. We therefore hypothesized that the host inflammatory response to VSV infection plays a critical role in the suppression of intratumoral VSV replication, and that by counteracting these responses we could substantially enhance VSV oncolysis and treatment efficacy.

Many inflammatory processes are mediated by chemo-attractant and immuno-modulatory molecules called chemokines, which play a central role in the host defense against invading viruses and in the pathogenesis of inflammatory diseases. A number of viruses have evolved elegant mechanisms to evade detection and subsequent destruction by various immune cells in the host.One such mechanism involves the production of secreted chemokine–binding proteins, which exhibit no sequence homology to any known host proteins, yet function to competitively bind and/or inhibit the interactions of chemokines with their cognate receptors, thereby suppressing the chemotaxis of inflammatory cells to the infection sites. While the functions and mechanisms of viral chemokine binding proteins (vCKBPs) have been extensively studied, they had not been exploited for the purpose of enhancing the oncolytic potency of heterologous viruses in cancer treatment. In this study we describe the molecular construction and characterization of a novel rVSV vector that encodes the secreted form of the equine herpes virus-1 glycoprotein G, which is a vCKBP that binds C, CC, and CXC chemokines with high affinity.

Using this vector we have shown in preliminary work (Figure 9) that it can effectively kill a variety of prostate cancer cells and are now beginning in vivo studies in immunocompetent mice to demonstrate efficacy – we hope to use this preliminary data and the knowledge from this grant to apply for future funding from the DoD using this vector.

Key Research Accomplishments

1. The enhanced killing abilities of VSV-F are mediated through syncytia formation without significantly diminishing VSV's strong induction of apoptosis.
2. VSV-F results in superior growth effects over that of wild-type VSV, resulting enhanced survival in vivo.
3. Prostate cancer cells display differential IFN response pathways, which can adversely its oncolytic capabilities.
4. The growth suppressive activities of VSV-F occur at doses of IFN which inhibit replication in IFN responsive cells.

Reportable Outcomes

Abstract at presented at the American Urological Association meeting: Vesicular Stomatitis Virus as Oncolytic Viral Gene Therapy for Prostate Cancer. Seth A. Strope, Jian Pu, Sherwin Zargaroff, Savio LC Woo, Simon J Hall, Abstract # 429.

Conclusions

The data clearly demonstrate the enhanced abilities of VSV-F, especially with repetitive injections, in a cell line which has retained some interferon sensitivity. We are currently exploring the role of the immune response as the underlying driver of this activity.

References

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